Stem cell chimerism in the germ cell lineage?

A polymorphic marker study

Annamari Alitalo
Faculty of Veterinary Medicine
Department of Basic Veterinary Sciences/ Anatomy
University of Helsinki
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To my parents

Abstract

Primordial germ cells are the embryonic precursor cells of sperm and ova. They are concluded to differentiate outside the gonadal area probably in the extraembryonic mesoderm. Their invasive migration through the stromal tissues of the dorsal mesentery adherent to the extracellular matrix is a well accepted fact in mice. The aim of this study was to set up a technique based on the use of individual-specific markers to test the hypothesis on the migration of primordial germ cells in a large animal model. The hypothesis, supported by feeble proof until now, states that there would be substantial chimerism in the progeny of primordial germ cells in bovine twins originating from transmission of the cells through placental vascular anastomoses during fetal life.

Our aim was pursued by microsatellite analysis of the highly polymorphic bovine MHC locus DRB3. Polymerase chain reaction and capillary electrophoresis were optimized using 21 non-chimeric animals, ten cows and eleven bulls. The DRB3 microsatellite of one bull was cloned to isolate single alleles. Whole blood-derived DNA from 11 chimeric animals, the leukocyte chimerims of which had earlier been quantified by *in situ* hybridisation, and 47 semen-derived DNA samples from twin borne AI bulls were analysed in the last phase of this study.

Primordial germ cell chimerism did not seem to occur in a substantial quantity in twin borne bovine bulls. Extension of the analysis described here to other polymorphic repeat loci should clarify the cases were traces of possible chimerism was found by analysing the above locus.

Key words: primordial germ cell, chimerism, chorionic vascular anastomosis, microsatellite

Kantasolu kimerismi ituradassa?

Polymorfisen markkerin tutkimus

Tiivistelmä

Primordiaaliset ituradan solut, niin sanotut PGC solut, ovat siittiöiden ja munasolujen sikiönkehityksen aikaisia edeltäjäsoluja. PGC solujen on päätelty erilaistuvan gonadialueen ulkopuolella, luultavasti sikiön ulkopuolella sijaitsevassa mesodermissä. Hiirellä näiden solujen invasiivinen vaellus suoliliepeen strooman läpi, soluväliaineeseen liittyneenä, on laajalti hyväksytty malli. Tämän tutkimuksen tavoite oli pystyttää yksilöspesifisiin markkereihin perustuva tekniikka jolla kyetttäisiin testaamaan hypoteesia PGC solujen vaelluksesta suureläin mallissa. Hypoteesin mukaan nauta kaksosilla esiintyisi pysyvää ituratakimerismiä PGC solujen siirtyessä sikiöstä toiseen istukan verisuonianastomoosien kautta.

Tavoitteeseen pyrittiin analysoimalla erittäin polymorfista naudan MHC-kompleksin lokusta; DRB3 geenin mikrosatelliittia. Polymeraasiketjureaktion ja kapillaari geelielektroforeesin optimointiin käytettiin 21:stä ei-kimeeristä eläintä; kymmentä lehmää ja yhtätoista sonnia. Yhden sonnin DRB3 mikrosatelliitti kloonattiin yksittäisten alleelien eristämiseksi. 11:sta, aikaisemmin *in situ* tekniikalla leukosyyttikimeeriseksi varmistetun, eläimen kokoverestä eristetyt DNA-näytteet ja 47:n keinosiemennykseen käytetyn, kaksosena syntyneen sonnin spermasta eristetyt DNA näytteet analysoitiin DRB3 mikrosatelliittialleelien lukumäärän suhteen.

PGC solu kimerismi osoittautui esiintyessään enintään marginaaliseksi ilmiöksi kaksoissonneilla. 47:n sonnin spermanäytteistä ainoastaan yhdestä löydettiin mahdollisia viitteitä neljästä DRB3 alleelista ja siten kimerismistä. Jatkotutkimuksissa pyritään täydentämään tekniikkaa käyttämällä useampia polymorfisia mikrosatelliittilokuksia analyysissä, jolloin suuremmalla todennäköisyydellä havaitaan normaaliin homo- tai heterotsygoottitilanteeseen nähden kasvanut alleelimäärä kimerismin esiintyessä.

avainsanat: primordiaaliset ituradan solut, kimerismi, suonikalvon verisuonianastomoosit, mikrosatelliitti

Stamcell chimärism i groddbanan?

En polymorfisk märkör undersökning

Sammanfattning

Primordiala groddceller är de embryonala föregångarna till spermier och ägg. Man har enats om att de utvecklas utanför den gonadala regionen och man tror att de differentierar sig i det extraembryonala mesodermet. Deras invasiva migration, fastbundna till det extracellulära matrixet genom det dorsala mesenteriets stromala vävnad, har blivit godkänd som fakta i en musmodell.
Syftet med denna undersökning var att hitta en teknik som baserar sig på individ specifika markörer för att testa en hypotes angående de embryonala groddcellernas vandring i en stordjursmodell.
Hypotesen, som fram till nu har stöttats med bara föga bevis, konstaterar att det skulle finnas substantiell chimärism bland descendenter från de primordiala groddcellerna i nötdjurstvillingar. Dessa härstammar från cellernas transmission genom vaskulära anastomoser i placenta under fostertiden.

Vi analyserade ett synnerligen polymorfiskt mikrosatellitlocus, DRB3, för att se mängden av olika genotyper i spermaprov. Optimeringen av metoden som baserar sig på PCR och kapillär elektrofores utfördes med 21 icke-chimära djur; tio kor och elva tjurar. DRB3 mikrosatelliten från en tjur var klonad för att isolera enskilda allel. Till sist analyserades sperman av 47 tjurar som är födda som tvillingar. Helblod av 11 djur som tidigare hade verifierats vara leukocytchimära med *in situ* hybridisation analyserades också.

Man kunde visa att de embryonala groddcellernas chimärism, förmodligen inte sker som ett substantiellt fenomen hos nötdjurstvillingar. En spridning av den nu använda metoden till andra polymorfiska repetition sekvens loci förklarar de tvivelaktiga fallen som hittades vid analysering av DRB3 locus.

Nyckelord: primordial groddcell, chimärism, chorions vaskulära anastomoser, mikrosatellit

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Abbreviations:

AI = artificial insemination

AP = alkaline phosphatase enzyme

BoLA = bovine lymphocyte antigen i.e. bovine MHC

bp = base pair

DRB3ms = DRB3 microsatellite

E1, E2 etc. = first embryonic day, second embryonic day etc.

ISH = *in situ* hybridisation

MHC = major histocompatibility complex

ms = microsatellite

PGC = primordial germ cell

SNP = single nucleotide polymorphism

SNP-PCR = single nucleotide polymorphism-PCR

SSEA-1 = stage-specific embryonic antigen 1

STR = short tandem repeat

STR-PCR = polymerase chain reaction using specific primers for a STR-locus

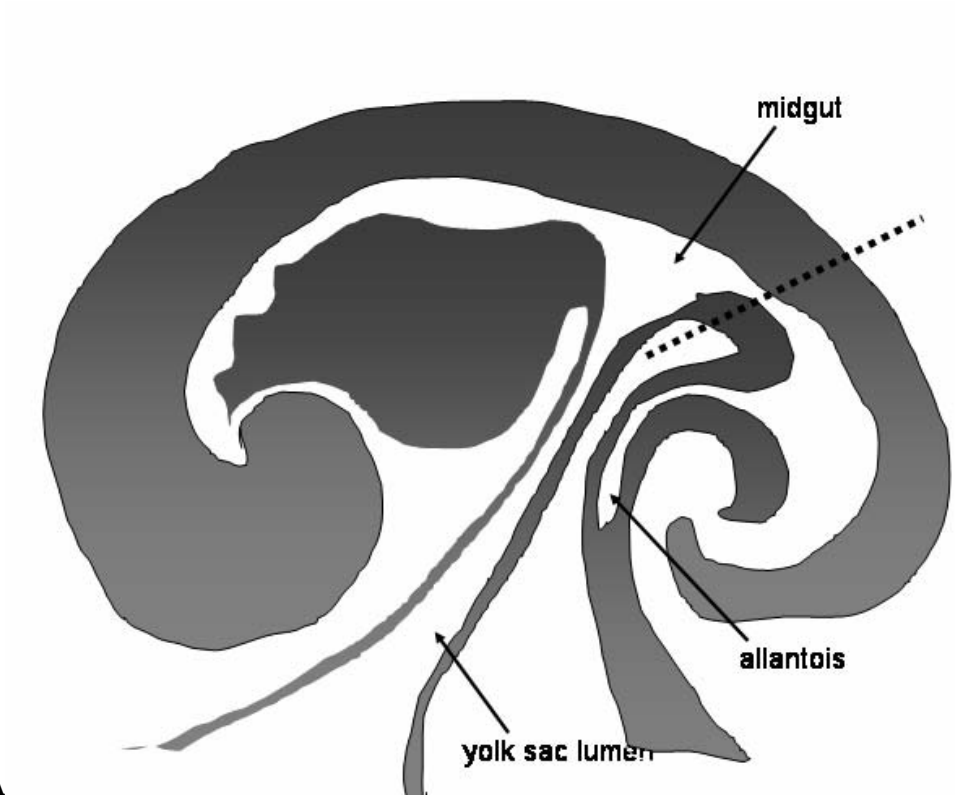
Y+ = Y-chromosome positive

Introduction

PRIMORDIAL GERM CELLS

Primordial germ cells (PGCs) are the embryonic progenitor cells of sperm and ova of the adult organism. In the bovine the first potential PGCs are identified in the caudal yolk sac of 18 day embryo by their alkaline phosphatase expression (Wrobel K-H and Süß F. 1998) (**Fig. 1**). As the embryonic disc folds most of PGC cells are carried into the developing mid- and hind-gut area immediately medial to the future gonad anlage by day 23-25. The mode of PGC migration from the gut area to the gonads is still a matter of dispute, not only in the mouse, but even more so in the less studied large animals (Curran et al. 1997, Freeman B. 2003 among others).

During embryonic folding the number of PGCs increases from approximately 500 to 1000 cells. At day 40, the bovine gonads have partially differentiated and at this time PGCs are found resident in the gonad. Primordial germ cells, having some properties of stem cells themselves, give rise to a generation of germline stem cells, the gonocytes or prospermatogonia, after settlement in the gonadal ridges of mammalian male embryos. These migrate to a location between the Sertoli cells at the onset of spermatogenesis and differentiate into self-renewing spermatogonia. Spermatogonia of different types are described as precursors to meiosis-undertaking spermatocytes in at least three competitive models. The outcome in these three models is the same; a haploid sperm cell differentiating from a haploid spermatid through spermiogenesis (Haifan 1997). Studies of PGC properties require that one deduces backwards along this path beginning at the very bottom, that is the sperm cells in the semen. The present study used such a retrospective analysis.



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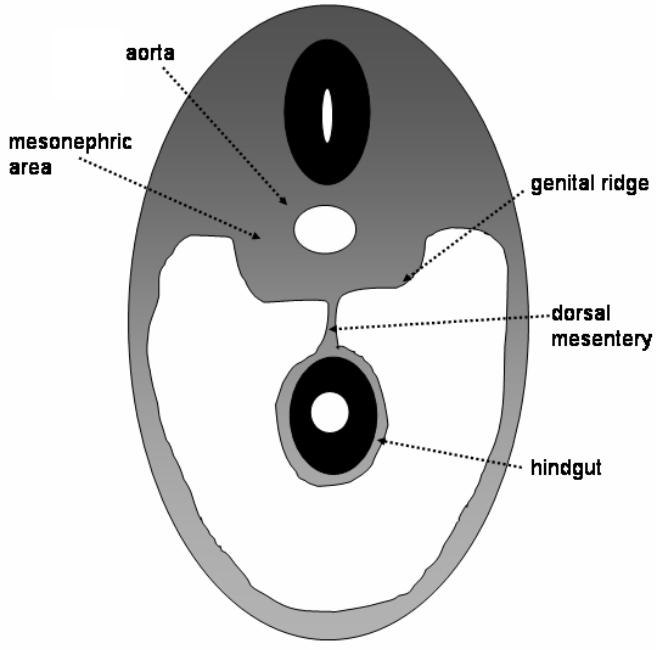


B

Fig. 1. Structures essential for PGC migration. A. A schematic presentation of a craniocaudally sectioned embryo. Cranial is left, dorsal up. **B.** Transversal section through the plane stippled in A. Dorsal is up.

MECHANISMS OF MIGRATION

First cytogenic evidence of germ cell chimerism in bovine twin calves of opposite sex was presented in 1962 (Ohno et al. 1962). An intravascular transportation model for bovine PGCs was subsequently presented. In 1965, the presence of alkaline phosphatase (AP) positive cells in the vasculature of bovine triplet embryos was suggested as an indication of possible PGC transmission through chorionic vascular anastomoses connecting bovine twins (Ohno et al. 1965). Somatic cells in the testis were not excluded as a source of the XX metaphase figures in the early studies on bovine germ cell chimerism (Ford and Evans 1977). Later studies showed that AP positivity is not a specific marker for the PGCs (Wrobel and Süß 1998). The AP positive cells found could be explained as bipotential stem cells of the hematopoietic lineage capable of differentiating into a PGC direction if located aberrantly as in embryomas, germinomas and teratomas (Wrobel and Süß 1998). Studies assessing AP positivity more critically support an extravascular migration of bovine PGCs (Jost and Prepin 1966). Even these studies have not excluded that minor contributions to the pool of spermatogonia in the bovine testis could originate from the PGCs lost into the bloodstream at some stage. Also embryonic folding has been thought by some to explain normal relocation of PGCs altogether (Wrobel and Süß 1998). The possible occurrence of germ cell chimerism due to intravascular transportation of bovine PGCs was the motivation of this study.

The most recent supportive evidence for the hypothesis of germ cell chimerism came from fluorescence *in situ* hybridisation (ISH) experiments with X-chromosome specific probes (Redjuch et al. 1998). These studies used testicular tissues of three bulls, which each were born twin to a sterile heifer, so called freemartin (Lillie 1917). A problem with such studies on the germ cell chimerism of freemartin twins is the absence of Y-chromosome specific sequences in the translocated donor cells of the XX karyotype. It is unlikely that XX cells can participate in the functional germ stem cell pool in the male gonadal environment (Ford and Evans 1977). This does not depend on migration ability since XX karyotype PGCs have been shown to successfully migrate into an experimentally produced XX genotype testis (Short 1970). Studies on chimeric mice indicate that only XY cells are able to undergo spermatogenesis (McLaren 1975). For this reason bull twins were used in this study, instead of sexually mismatched twins.

PGCS THROUGH ANIMAL PHYLA

Flies, worms, frogs, fishes and birds

Some basic knowledge on PGC biology is obviously needed for the evaluation of the early studies on their chimerism.. Allocation to the germline in mammalian species is not predetermined, as shown by cell transplantation and retroviral marking studies. In contrast, allocation is induced in some site-dependent manner just prior to gastrulation (Gomperts 1994b). One theory is that PGCs are sheltered by their established, transient location outside of the embryo at a time when extensive DNA methylation is underway in the somatic, embryonic cells.

In other species, germ line allocation is differently regulated. PGC determination is maternal in the fly *Drosophila melanogaster*, the worm *Caenorhabditis elegans*, the frog *Xenopus laevis* and in zebrafish *Danio rerio* where a distinquishable region of the ovum is loaded with mRNA transcripts of cell fate determining genes (Starz-Gaiano and Lehmann 2001, Haifan 1997). Yet, in these species as well as in mammals, the PGCs end up in the gut region after gastrulation (Buehr 1997).

In avians PGC transportation is partially intravascular. Chick PGC precursors arise in the epiblast and move to the hypoblast prior to gastrulation. They are pushed outside of the embryo proper with the hypoblast cells as these move aside incoming endoderm cells. In their extraembryonic locations, ranging from the ventral hypoblast to diffuse locations among the blood vessel forming mesodermal mesenchymal cells, PGCs circumvent gastrulation (Ginsburg 1997). Subsequent intravascular transportation of the PGCs back to the embryo proper and their adherence to the gonadal anlage occurs after gastrulation. The occurrence of intravascular PGCs, totipotent cells as they are, is taken advantage of in the production of chimeric and transgenic chickens (Il-Kuk et al. 1997).

Mice and cattle

The phases of PGC translocation are best studied in murine embryos owing to the scarcity of these cells in lower vertebrate models and the long generation times of larger animals. In mice the determination of PGCs is probably linked to their localization in the proximal epiblast region adjacent to the extraembryonic ectoderm. The extraembryonic ectoderm expresses the paracrine factors BMP4 and BMP8b, which possibly play an inductive role (Bendel-Stenzel M. et al. 1998). Autografting studies on mouse embryos have shown the potency of any epiblast cell to participate in the germ lineage (Tam 1996). All epiblast cells express Oct-4 transcription factor which is related to the maintenance of pluripotency. This is the only gene yet identified to be expressed solely in

PGCs after gastrulation (Watson 2001). In mice the PGCs are found at embryonic day 7 in the hindgut endoderm similarly to the bovine PGCs around embryonic day 24. The active migration of murine PGCs begins at E9. Pioneers of the approximately 100 PGCs in the hindgut area reach the mesonephric area and the sexually indifferent gonads at E10.5, before elongation of the dorsal mesentery (Gomperts et al.. 1994a). Most of the PGCs have reached the genital ridges after invasion through mesodermal tissues of the elongating dorsal mesentery by E11.5, forming a network of ca 25 000 cells progressively connected to each other via cytoplasmic processes along the migration pathway. The migrating cells have been shown to adhere to the extracellular matrix proteins laminin and fibronectin and to regulate the strength of this adhesion in accordance with the phase of migration (Garcia-Castro et al.. 1997).

The distance from the hindgut through the dorsal mesentery to the genital ridge has been measured to be 10-50 µm in the mouse embryo of E9.5 at the time of PGC migration (Wartenberg 1983). This distance is some 1 mm in the bovine embryo at 8 mm crown-rump length (i.e. approximately 27 day embryo according to Winters et al. 1942 and Rüsse 1991). Thus it has been claimed that if these cells are able to migrate actively in the mouse, the 20 to 100 times longer path required in the bovine would demand some other mode of translocation (Wartenberg 1983, Wrobel and Süß 1998).

THE MODEL; BOVINE TWINS

Already in 1945 immunological tolerance in skin grafting experiments between bovine twins resulting from permanent leukocyte chimerism was found (Brent 1997). These are a consequence of placental vascular anastomosis causing union of blood circulations of the twin embryos and therefore exchange of material including hormones and circulating cells during development *in utero.* In sexually mismatched bovine twins the XX genotype animal, the so called freemartin, is masculinised to a varying degree as it is influenced by hormones derived from the XY genotype animal prior to sexual differentiation (Vigier et al. 1976). This phenomenon known is as the freemartin effect. Previously the level of chimerism in different freemartin tissues has been studied in our group by ISH using a Y-chromosome specific probe. These studies have revealed on average less than one percent Y^+ cells in non-hematopoietic tissues (Niku et al., in press).

The placental vascular anastomoses provide the basis for the verified freemartin effect in ca 92% of bovine heterosexual twins (Lillie 1917). These anastomoses are established when the avascular chorionic and finally the vascularized allantoic membranes of the two embryos fuse forming a single, united chorioallantoic membrane (Hafez and Rajakoski 1964). The chorions have been

observed to connect embryos of approximately 10 mm crown-rump length, while the allantoes fuse with the chorions at ca 15 mm stage (Lillie 1917). The earliest observations on chorionic vascular anastomosis between bovine twins are from embryonic day 30, which according to measurements is roughly equal to 10 mm crown-rump length (Hafez and Rajakoski 1964). Whether PGC migration is still undergoing at this stage is not clear yet.

PGC HISTOCHEMISTRY

The limitations imposed by lack of cell specific markers have made histochemical studies on PGC migration challenging. Jost and Prepin (1966) showed that AP was an unspecific marker of bovine PGCs at embryonic days 25 to 34 as some intravascular, probably hematopoietic, cells were also positive at this stage. 28 lectins in combination with AP have been studied to identify bovine PGC markers at embryonic days 18 to 39. Of these three were found to bee moderately PGC specific (Wrobel and Süß 1998).

One of the most essential murine PGC markers has also been studied in bovine embryos with varying results. Stage-specific embryonic antigen 1 (SSEA-1) has been used as a marker for undifferentiated, pluripotential embryonic germ (EG) cells and migratory PGCs in the mouse (McLaren and Durcova-Hills 2001). SSEA-1, a cell surface glycoprotein, is also considered a candidate involved in the adhesion of germ cells to the extracellular matrix during migration (Gomperts 1994b). Bovine SSEA-1 was not recognized by the antibody recognizing a mouse, human and pig SSEA-1 epitope in immunohistochemical studies on 34, 37 and 40 day bovine embryos (Choi et al. 1999). Thus SSEA-1 is not a useful PGC marker in the bovine. A negative staining result could be due to a different SSEA-1 structure on bovine PGCs which the antibody used does not recognize and such result could also result from the absence of SSEA-1 on bovine PGCs.

As these limitations are not yet overcome, immunohistochemical studies of bovine PGC migration are limited. This is one of the reasons why a retrospective approach on this issue could be more informative. The genotyping of semen DNA could give indications on chimerism among PGC precursors if the donated germ cells undergo spermatogenesis efficiently in the recipient testis.

A POLYMORPHIC MARKER STUDY

Short tandem repeat (STR) analysis from bulls used for artificial insemination (AI) provided a comparably facile method for evaluation of sperm chimerism (**Fig. 2**). In this analysis method a polymorphic STR locus is amplified by PCR. Detection of more than two alleles of different length in the subsequent electrophoresis of PCR products is an indication of chimerism. Semen samples were easily derived, DNA extraction was straightforward and use of nonradioactively labeled, i.e. fluorescent, primers for PCR and subsequent capillary electrophoresis was possible. Contrary to other methods based on polymorphic loci, such as the use of sequence specific PCR primers or real-time SNP-PCR (single nucleotide polymorphism PCR), haplotyping is not a prerequisite for STR-PCR. Sensitivity is also a major factor. Denaturing gradient gel electrophoresis (DGGE) and single stranded conformational polymorphism (SSCP) are also techniques which allow quantification of polymorphic alleles. However these were ruled out as possibly less sensitive techniques. STR-PCR has some limitations on allele lengths etc. when used in a quantitative study on chimerism. It also lacks full quantitative accuracy especially when coupled to electrical injection of the sample into a capillary device (Kleeberger et al. 2003). Electrophoresis on slab gels gives a more accurate quantification, but quantification with this method is haplotype specific due to length and sequence specific amplification differences of the alleles in the PCR (Scharf et al. 1995).

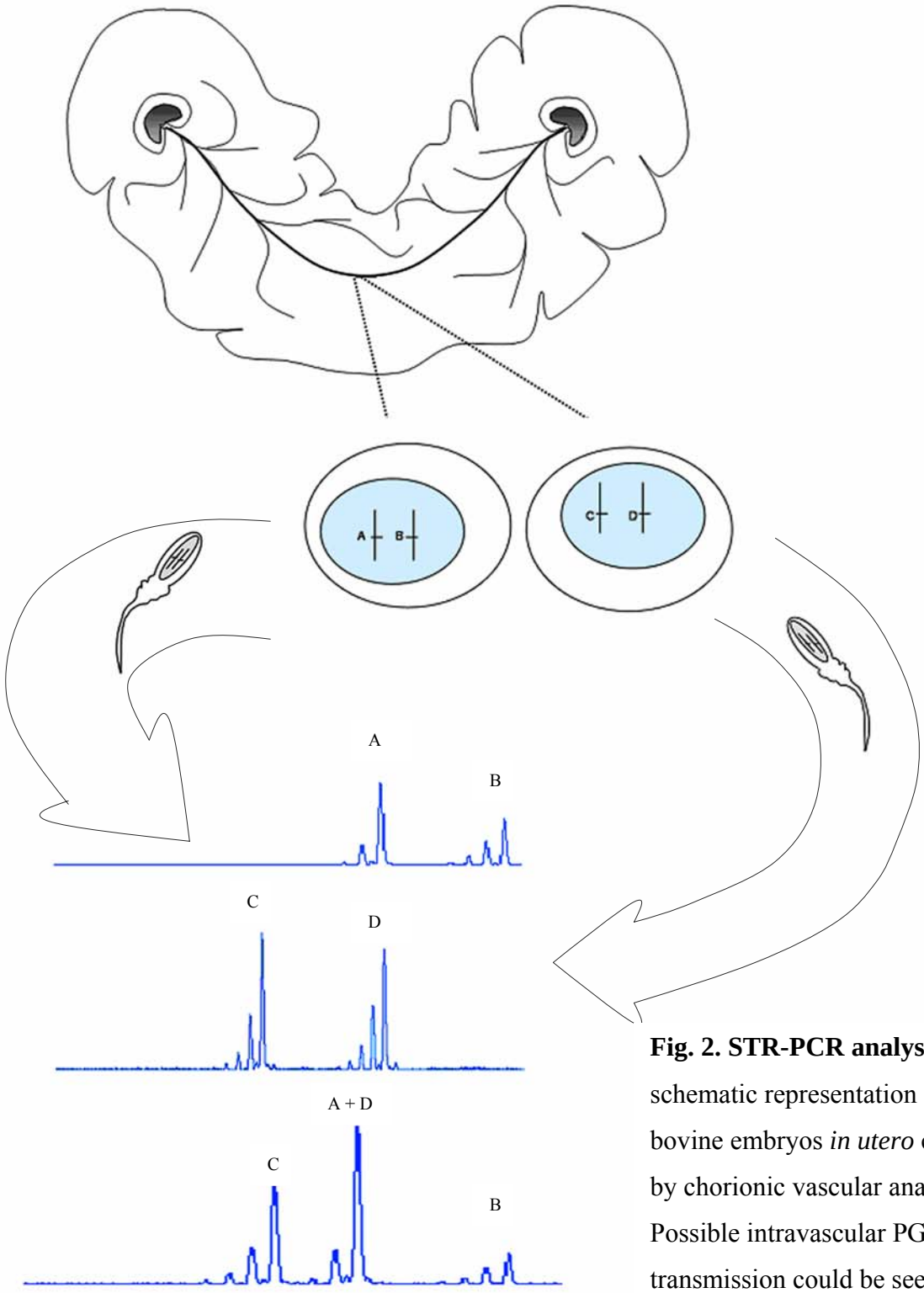


Fig. 2. STR-PCR analysis. A schematic representation of two bovine embryos *in utero* connected by chorionic vascular anastomoses. Possible intravascular PGC transmission could be seen in STR analysis of semen. The chimeric STR pattern is composed of two genotypes.

BOVINE LEUKOCYTE ANTIGEN

A microsatellite (ms) located in the BoLA (bovine leukocyte antigen) region on chromosome 23 was chosen as the first locus used in the STR-analysis. BoLA is the bovine homolog of human MHC and thus includes the genes coding for MHCI and MHCII heterocomplexes. These glycoprotein complexes in turn bind pathogen derived peptides and display them on the cell surface. As a result of evolutionary competiton between pathogen and host mutation rates, MHC is possibly the most polymorphic region of mammalian genomes and as such a welcome tool for our studies (Lewin 1996). The high degree of polymorphism and wide range (149-207 bp) of allele lengths made DRB3 intron 2 ms the STR of first choice from at least 11 mss located in the BoLA (Lewin 1996). DRB3 could be the most significant of the bovine MHC IIß genes as it is expressed at a high level on peripheral lymphocytes (Ellengren et al. 1993). Exon 1 of the gene encodes a part of the antigen recognition site (ARS) of MHC II (Sigurdardóttir et al. 1991). DRB3 intron 2 ms (DRB3ms) is well characterised (Ellengren et al. 1993) with 25 alleles discriminated (van Haeringen et al. 1999) and provides a relatively good tool for typing the closely associated, highly polymorphic DRB3 exon 1 (Sigurdardóttir et al. 1991). After retrieving ms haplotype information, chimeric samples could be quantified most accurately by SNP-PCR using DRB3 exon 1 SNPs. Selecting for a sufficiently polymorphic SNP to be used requires information on the haplotypes in the sample. The resolution of DRB3 exon 1 typing by analysing linked STR loci is refined when DRBP1, a MHC IIβ, ms is analysed in addition to DRB3ms (van Haeringen et al. 1999).

Besides a highly polymorphic tool, the BoLA haplotypes of chimeric animals could be of interest as such. One could ask what the consequences are of an increased number of peptides bound on cell surfaces of a chimeric leukocyte pool on the immune resistance of the animal. The antigenic pathogen derived peptides mounting immune responses are bound on the ARS of MHC heterocomplexes. An increased repertoire of ARS sequences of a hematopoietic chimera could mean a surplus of high affinity bound pathogen derived peptides leading to superior resistance. Direct associations between resistance to infectious diseases and BoLA haplotypes have been difficult to prove since non-genetic factors play a major role in incidences of these diseases. As an example of the concurrent and tangled roles of BoLA loci in the heritance of resistance, Lunden et al. (1990) have shown significant association between an MHC II haplotype (DQ^{1A}) and clinical mastitis but this relationship could as well have stemmed from DRB3 haplotype DRB^{1A} which coincidenced with the DQ^{1A} haplotype.

Materials and methods

OPTIMIZATION OF STR-PCR

Genomic DNA was isolated from blood samples of 10 non-chimeric cows standing at Viikki Research Farm, University of Helsinki and sperm samples of 11 non-chimeric bulls used for artificial insemination (AI) at Sisämaan Jalostus, Hollola, Finland. DRB3 exon 2 ms was analysed as follows. PCR was carried out in 20 µl volumes using a primer mix of unlabeled LA53 or 5´-biotin labeled LA53 (5 pmol), LA54 (3 pmol) and FAM-labeled LA54 (2 pmol) (Proligo France SAS). These primer sequences were obtained from the International Society for Animal Genetics BoLA Committee web site (original article from Ellegren et al. 1993 containing an error). The LA53 primer was shortened from the 5´end by redundant, unspecific 5 nucleotides (to 24 nt), destroying an EcoRI restriction site.

The sequences of the primers are LA54: 5'-GAGAGTTTCACTGTGCAG-3' and modified LA53 (24 nt): 5'-ATTCCCGAGTGAGTGAAGTATCT-3'.

The products of ten PCR reactions were sequenced and these primers showed specific amplification of the desired locus. All laboratory work was done with special emphasis on preventing sample cross-contaminations.

Different template concentrations (6-50 ng/20 µl), polymerases PFU (0.5U; Promega), Taq based DyNAzyme II (0.4U; Finnzymes) and PHUSION (0,2U; Finnzymes), temperature profiles and cycle numbers (17, 20, 26 and 30) were used when testing for optimal amplification conditions. The temperature profiles tested are given in **Table 1**. The dNTP concentration was held constant at 200 µM. The Taq-based polymerase was tested with Mg^{2+}-concentrations ranging from 0.5 mM to 2.5 mM and in 1.5 mM Mg^{2+} with 1 M or 1.4 M betaine (annealing temperatures 44-50°C).

PFU			DyNAzyme II	PHUSION
95°C, 1min	95°C, 2min	95°C, 2min	95°C, 2min	98°C, 2min
95°C, 60s	95°C, 60s	95°C, 45s	95°C, 45s	98°C, 8s
50°C, 30s	50°C, 30s	50°C, 30s	50°C, 30s	50°C, 30s
72°C, 60s	72°C, 60s	72°C, 60s	72°C, 60s	72°C, 7s
72°C, 5min	72°C, 5min	72°C, 5min	72°C, 5min	72°C, 5min

Table 1. The temperature profiles tested for the above polymerases. After initial denaturations of 2min. 30 cycles were performed followed by a final extension of 5 minutes.

8 µl of the PCR product was first checked for successful amplification on agarose gel (3% NuSieve 3:1 agarose, BioWhittaker, Rockland, USA) and the remainder was filtered through a polystyrene membrane (Montage PCRµ96, Millipore, Mass., USA) rehydrated in 30 µl water and mixed with 10 µl formamide and a size ladder (GeneScan®-500 [ROX]™, Applied Biosystems, Foster City, USA). 2 µl of this mixture was used for capillary electrophoresis (ABI 3100, POP-6™ polymer, Applied Biosystems, Foster City, USA).

5´Biotin label on LA53 (24 nt) primer was used to confirm that it is full length i.e. the absence of any discrepancy caused by short-cut oligonucleotide synthesis. The biotinylated PCR product was incubated in room temperature with avidin coated polystyrene beads (5 µl 5% v/w, prewashed with binding solution, BS, 10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5) for 10 min and unlabeled products were removed by washing twice through a 0.45 µm filter with 50 µl BS. The fluorescently (FAM) labeled strand was denatured by applying 30 µl of formamide, incubating 5 min and centrifugation through the filter. It was then mixed with the size standard and electrophoresed. Results were analysed with ABI Prism® GeneScan Analysis Software.

CLONING

To isolate single alleles a DNA sample from a heterozygous control bull was subjected to PCR in 50 µl volume using unlabeled LA53 (24 nt) and LA54 primers and DyNAzyme II polymerase. The PCR products were purified from agarose gel and cloned using a cloning kit (TOPO-TA with

pCRII-TOPO vector and chemically competent TOP10 cells, Invitrogen Ltd.), according to the manufacturer´s recommendations. The plasmids from eight single colonies were purified, dideoxy-sequenced (using AmpliTaq DNA Polymerase FS, Applied Biosystems) using vector primers and the ms lengths were calculated. Two colonies representing different allelic variants of the ms were selected and further grown, subjected to larger scale plasmid purification and titrated as templates in the PCR reaction (total 10 pg of plasmid template in 20 µl PCR-reaction) using the same three enzymes and four primers in subsequent trials as in optimizing for genomic DNA. Since quantification of the amount of template was essential, spectrophotometric measurements were repeated thrice.

MICROSCOPY

Some of the semen samples from non-chimeric control animals we also used for evaluating the proportions of somatic cells and sperm cells in semen. This was done both by direct microscopy of unstained samples and samples stained manually or automatically with the May-Grünwald-Giemsa (MGG) solutions (metanol fixation used in both).

EVALUATION OF CHIMERISM

After DNA isolation from 62 frozen AI semen samples and two fresh semen samples and the PCR optimization trials described above, genomic DNA samples from 63 twin bulls were subjected to our STR-PCR analysis. Data on the bulls were obtained from The Finnish Animal Breeding Association (FABA) records All of these bulls, except for the two from which fresh samples were obtained, had already been slaughtered. Also DNA isolated from blood samples of 12 sexually mismatched twins were analysed to be compared to the semen samples. This group of 12 animals comprised of five bull-freemartin twin pairs and one freemartin. The total leukocyte chimerism of these animals had earlier been quantified in our laboratory by ISH using a Y-chromosome specific probe (Pessa-Morikawa et al. 2004).

Results and discussion

OPTIMIZATION OF STR-PCR

All template concentrations tested (6-50 ng/20 ul) showed adequate amplification in the PCR. In contrary to 30 cycles, 17, 20 and 26 cycles amplified the template insufficiently. An initial denaturation of 2min. at 95˚C and subsequent 45s denaturations at 95˚C in each cycle were optimal for PFU and Taq polymerases.

Taq polymerase adds an adenine nucleotide to the 3´end of PCR products with a sequence dependent activity. This was at first thought to cause confusion in the STR-PCR analysis. The first PCR reactions were done with PFU polymerase which has no 3´adding activity. Several amplification products per allele were observed, which was unexpected, and thus this enzyme was compared with a Taq based enzyme (DyNAzyme II) and a Pyrococcus like enzyme with fused dsDNA binding domain (PHUSION). PFU produced approximately five fragments with one nucleotide length differences per allele. The pattern created by Taq polymerase was more distinct with commonly length differences of two nucleotides. Supposedly this could have been caused by PFU´s proofreading activity, which Taq lacks. Later a new Pyrococcus like enzyme (PHUSION), which is also a proofreading enzyme, was tested. It proved unsuitable, giving the greatest number of peaks in electropherograms. The peaks again differed most commonly by one nucleotide in length. The poor results obtained using this enzyme may have been, at least partially, influenced by its high productivity; it was also noticed in other experiments that overloading of the PCR product into the capillary caused splitting of the peaks leading into the stutter pattern having a substantial amplitude.

The presence of multiple PCR products was suspected to arise from a 5´-deficient unlabeled primer. A newly ordered LA53 (24nt) primer had no obvious effect on the outcome. For the same reason biotin labeled LA53 was tested. This proved useful. Besides removing any discrepancy caused by the primer, it made the pre-electrophoresis filtration protocol more effective which may have concentrated the product. It was known that the membrane originally used for pre-electrophoresis filtration gives about 70 % yields for under 300 bp fragments.

Once Taq polymerase was chosen as the enzyme to be used in subsequent experiments, different reaction conditions were tested for it. The pre-optimized buffer for Taq contained 10 mM Tris-HCl, 1.5 mM MgCl, 50 mM KCl, 0.1% Triton X-100, pH 8.8 (25°C). Experiments to improve the Taq-polymerase function by Mg^{2+} optimization or betaine addition did not result in significant improvement when compared to use of this buffer. The Mg^{2+} concentrations tested and the results obtained are shown in **Table 2**. The 1.0 M and 1.4 M betaine concentrations tested with annealing temperatures ranging from 44°C to 50°C even inhibited the reaction. Possibly a lower betaine concentration would have provided better results (not tested).

Mg^{2+} concentration	0.5 mM	0.75 mM	1.0 mM	1.25 mM	1.5 mM(*	1.75 mM	2.0 mM	2.25 mM	2.5 mM
amplification	-	-	-	+	++	+	+	-	-

Table 2. Taq-polymerase function in the Mg^{2+} concentrations tested.

- = no amplification observed

+ = some amplification

++ = efficient amplification

* = pre-optimized buffer

Optimization resulted in a STR-PCR protocol with 30 cycles, Taq polymerase (0.4 U), 200 uM dNTP, the pre-optimized buffer described above, 25 nanograms of template and biotinylated LA53(24 nt) primer (5 pmol) together with FAM labeled (2 pmol) and unlabeled (3 pmol) LA54 primer in 20 ul.

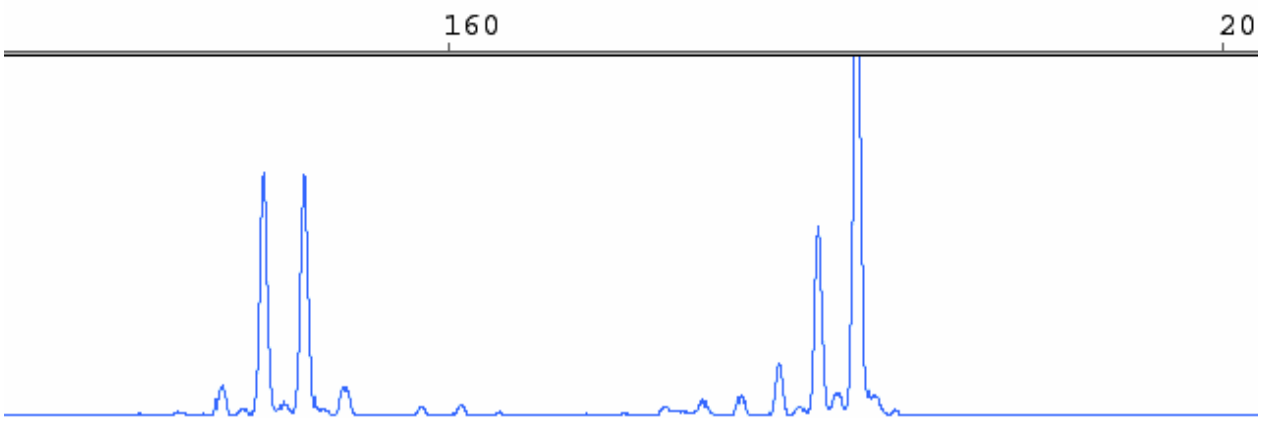
SENSITIVITY

The cloned alleles were also used for obtaining indications on the sensitivity of the technique. This was done by mixing the plasmids containing the cloned and so isolated alleles in known ratios and using these mixtures as templates in the PCR reaction. The biotinylated primer was used in these experiments. The percentages of the 149 bp and 183 bp long alleles are shown in **Table 3**. The allele lengths were obtained by sequencing.

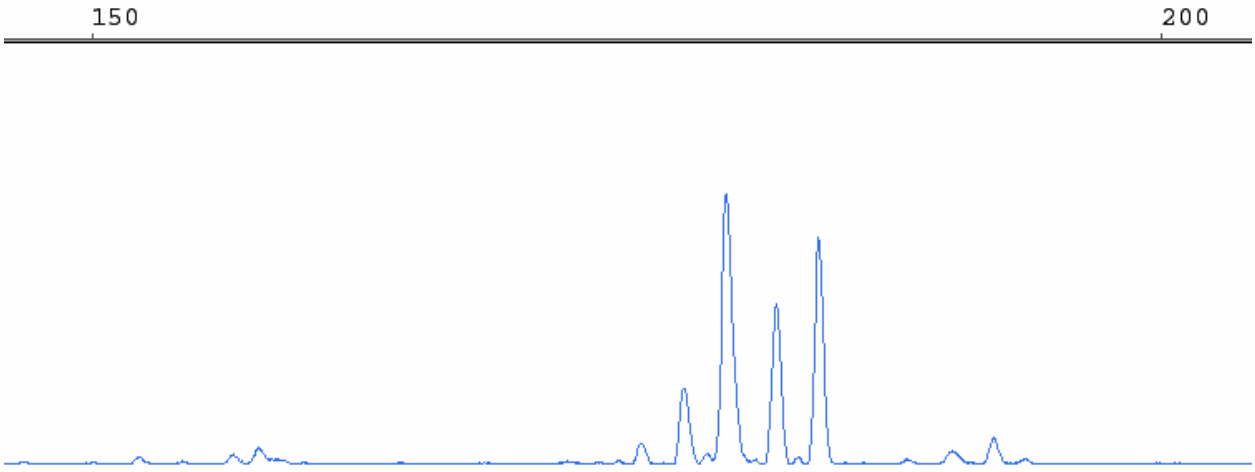
149 bp (%)	183 bp (%)
0	100
1	99
3	97
5	95
10	90
15	85
20	80
25	75
30	70
40	60
50	50
60	40
70	30
75	25
80	20
85	15
90	10
95	5
97	3
99	1
100	0

Table 3. Percentages of isolated alleles in the template mixtures used when estimating the sensitivity of the technique. A total of 10 pg plasmid

Both Taq based enzymes showed detectable amplification of the 183 bp allele when it comprised 3% (97% 149 bp allele) or more of the total template amount. PFU amplified this allele sufficiently only in 25% relative template quantity in this set up. The titration was carried out only once so random deviations caused by pipetting etc. may play a role in these results. Cross contamination by the shorter allele, competing as a template, could be seen in the 100% 183 bp (0% 149 bp) sample. Some cross contamination could also have occurred in other samples where the 183 bp allele comprised less than 50% of the total template amount (**Fig. 3A**).



A



B

Fig. 3. Two typical allele patterns obtained in DRB3ms analysis. A. Here the cloned 149 bp and 183 bp allele templates are analysed in a 3:97 ratio in the PCR reaction. Note the 160 bp and 200 bp marks on the upper margin. A 20 µl PCR-reaction was used with a total of 10 picograms plasmid template. **B.** Electropherogram of bull S4 showing definite patterns of 180 bp and 184 bp DRB3 intron 2 ms alleles and more dubious peaks at 158 bp and 192 bp (150 bp and 200 bp marks on the upper margin).

This contamination was probably minor compared with the applied template amount. The surface area of the 149 bp allele was smaller than the surface area of the 183 bp allele up to a 25%/75% ratio, respectively. In greater ratios concentrations the area relation rapidly inverted as the shorter

allele was preferably amplified in the PCR. It could also be speculated that the shorter fragment entered the electrophoresis capillary more effectively because of smaller size. The contamination could also have arisen from colony selection, plasmid purification or the PCR. Sequencing results were contradicting to the first possibility. Agarose gel electrophoresis of the PCR products with differing template relations showed an expected range of band intensities **(Fig. 4)**. The sensitivity of STR-PCR is probably dependent on allele length and sequence. A reconstruction graph for quantifying chimerism by STR-PCR would use genomic DNA from two haplotype mismatching non-chimeric animals as templates. This interpolation between known data points, is described in Kleeberger et al. (2003). Such a reconstruction graph can be tested by analysis of freemartins earlier quantified by a comparable method, ISH. Isolation and analysis of alleles is advisable since confirms the appearance of a pattern created by a single allele. Partially superimposed allele patterns can then be discriminated.

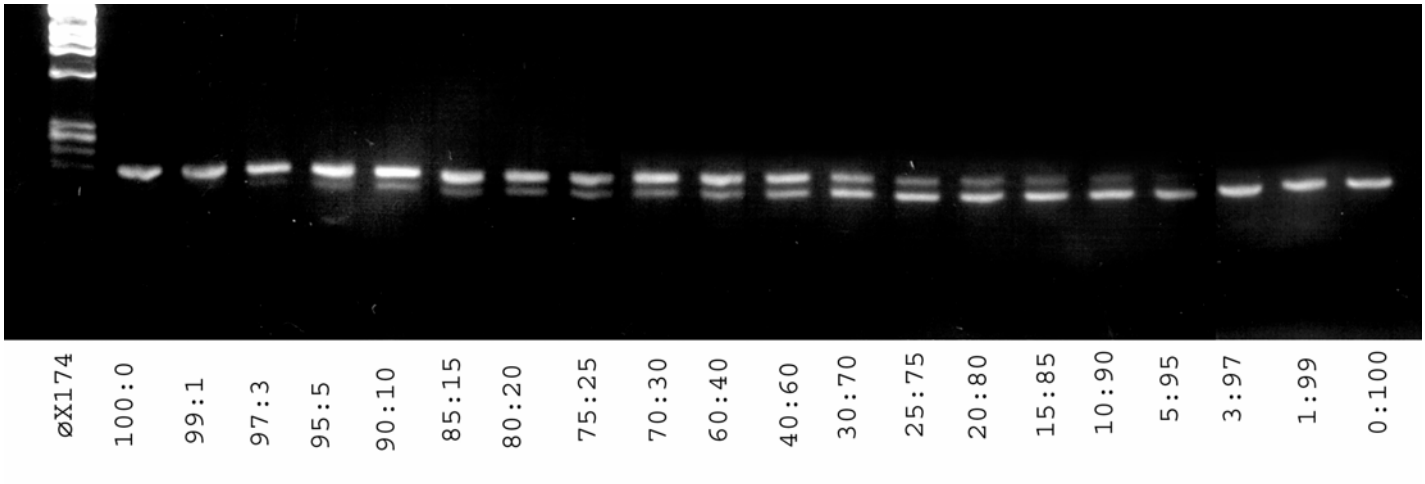


Fig. 4. Band intensities obtained with varying relative template concentations in DRB3 PCR. øX174 is the size standard used (øX174 DNA - *Hae* III Digest, Finnzymes Finland). Allele template ratios (183 bp/149 bp) in each sample are under the figure.

SOMATIC CELLS

Accurate estimation of somatic and germ cell proportions in semen was impossible since MGG-staining did not stain the sperm cells well. Direct microscopy of unstained semen showed only a small percentage of somatic cell contamination. Some somatic cells could have passed unobserved, since they were less apparent than sperm cells when the sample was not stained. No substantial differences between the bulls were observed in these counts.

EVALUATION OF CHIMERISM

After refining the STR-PCR method as described above the chimerism of 47 semen samples from twin borne bulls was assayed. In the STR-PCR analysis of 46 bulls one or two clear allele patterns were seen. 14 samples of the initial 61 (marked S3 to S64) failed to show adequate fluorescence signal in their electropherograms. This was probably because of either unsuccessful PCR or injection into the capillary. The amount of template in 6 of these 61 samples in the first trials was in the order of only 10 ng (less than half of the normal 25 ng) due to minute amounts of DNA obtained from purification, leading to a fairly dilute concentration.

Analysis of the peak amplitudes, i.e. signal intensities, is essential in evaluating the possibility of chimerism based on STR analysis. An adequate amount of PCR product in the capillary makes detection of rare fragments in the sample possible. Overloading can cause multiplication of peaks representing single alleles and possibly blockage in the injection. In 33 runs, the amplitude of the major peak was over 600 units, which was considered sufficient for the detection of substantial chimerism. In particular, one sample, S4, had an ideal amplitude of 1000-1300 units for the two alleles of the individual (180 and 184 bp) (**Fig. 3B**). In addition, two possible peaks flanked these (at 158 and 192 bp), suggesting two other allele patterns. These patterns lie in the region of 143-215 base pairs as described for DRB3ms lengths (van Haeringen et al. 1999). The patterns are also redolent of the ladders seen for individual alleles. In S20 the area of the shorter (189 bp) allele peak was 4.5 times that of the longer (192 bp) and in S6 the ratio of a 186 bp allele to a 193 allele was 2.8/1. Expected competitive PCR amplification between the alleles could cause these observations. This is a potent explanation since the shorter alleles were in excess. Alternatively, an uneven injection into the capillary could have caused these ratios or they could have stemmed from a 1/3 relationship of the alleles in the DNA samples analysed. This option corresponds to the situation in a homozygous-heterozygous genotype of a chimeric animal. These speculations will be further tested by STR-PCR of other highly polymorphic markers.

The results obtained with the semen samples were supported by analysis on five bulls and their freemartin twins plus one additional freemartin, FM 62 (**Fig. 5**).

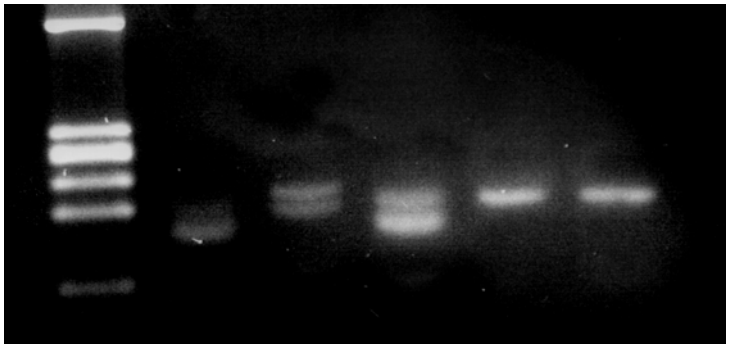


Fig. 5. The DRB3 alleles of freemartin heifers and their twins. From left to right: standard (øX174 DNA - *Hae* III Digest, Finnzymes Finland), freemartin FM96, twin of freemartin FM92, twin of FM96, freemartin FM56 and twin of FM56. See Table 2 for quantitative data.

The total leukocyte chimerims in these individuals ranged from 33 to 85% Y+ in ISH. In only one pair (FM96, FM96B) and FM62 three alleles were detected (**Fig. 6**). In the other four twins only one or two allele patterns were seen. A drastic deviation from a 1:1 ratio of the surfaces areas of the alleles detected would indicate homozygote-heterozygote chimera. The surface area ratios of the alleles seen in these electropherograms ranged from 0.93 to 1.03. Thus complete sharing of alleles or a homozygote-homozygote composition of genotypes is presumed. The DRB3ms lengths and leukocyte chimerism of these freemartins and their twins are reported in **Table 4**.

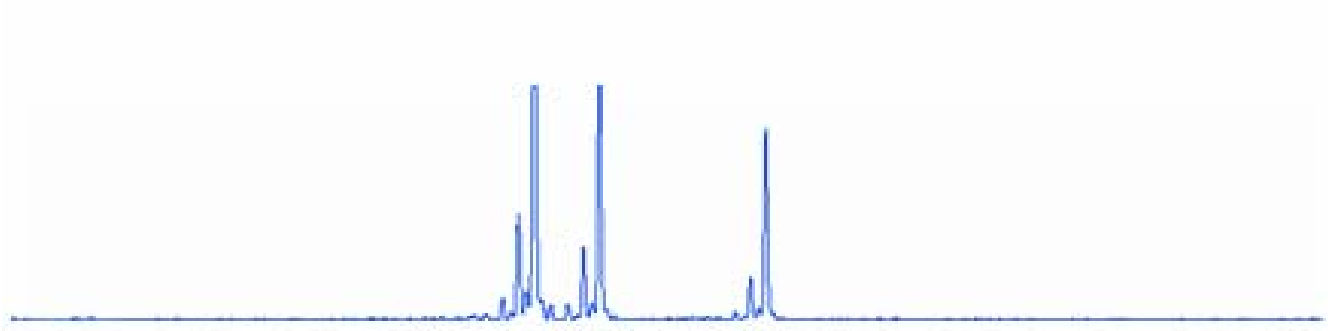


Fig. 6. STR-PCR analysis of FM96. Three DRB3 alleles (160 bp, 168 bp and 189 bp) were typed. 47% of leukocytes of this chimeric animal were Y^+ in ISH.

animal	DRB3 length [1 (bp)	Y^+ cells in total leukocytes (%)
FM 56 and	189(/191[2)	74
FM56B	- ll -	80
FM96 and	160/168/189	47
FM96B	- ll -	33
FM92 and	182/203	65
FM92B	- ll -	67
FM83 and	186/203	61 [3
FM83B	- ll -	64 [3
FM93 and	180/189	85
FM93B	- ll -	
FM62	160/180/189	75

Table 4. The DRB3ms alleles and leukocyte chimerism of six freemartins and their brothers (B). Y^+ cells total leukocytes quantified by ISH (Pessa-Morikawa et al. 2004).
[1 has to be interpreted as ±2 bp, see text for additional description
[2 homo- or heterozygosity could not bee ascertained
[3 percentage Y^+ T-cells

INFORMATION GAINED ON BOLA HAPLOTYPES

Despite use of an internal size standard, the apparent DRB3ms lengths of individual animals varied up to 2 nucleotides in successive electrophoretic runs. Comparing allele length frequences with results obtained in other laboratories would require accurate length determination. This is usually done by running a pre-haplotyped sample alongside the analysed sample in the capillary. An alternative is to run the analysed sample alone a number of times to reach a sufficient level of reliability. Since most of our samples have this far been run only once and no pre-haplotyped sample has been used, the lengths of single alleles have to bee interpreted as ± 2 nucleotides.

Of a total of 79 animals analysed, the DRB3 haplotype could be determined in 74. In 5 individuals the allele pattern was unclear. These could have been heterozygotes with two nucleotide length

differences between the alleles or homozygotes. Only four animals of the 74 typed were classified as definite homozygotes.

The allele length incidences found in our panel of 74 typed animals are summarised in **Fig. 6**. Excluding the five somewhat unclear cases from the panel would shift results towards a greater heterozygosity value, i.e. 94.6% without these vs. 88.6% if these were taken in as homozygotes.

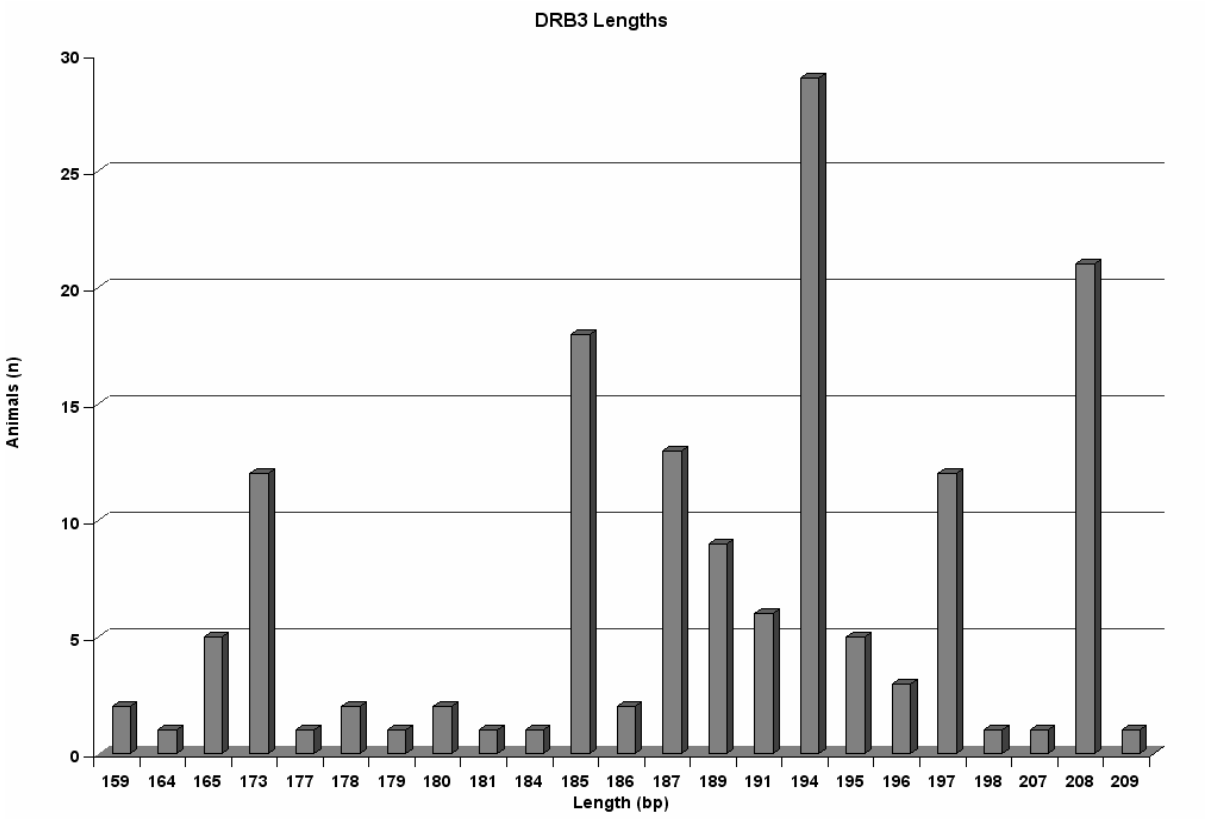


Fig. 6. The DRB3 intron 2 ms lengths of 74 animals typed. LA54 and modified LA53(24nt) primers were used in STR-analysis and five nucleotides were added to the obtained lengths to correspond to the results obtained with unmodified (29 nt) LA53 primer.

If the consequences of leukocyte chimerism on the immune resistance of the animal were to be studied, assuring an actual increase in the repertoire of ARS sequences is needed. DRB3ms is useful in selection of chimeric animals in such a study. This is due to close linkage to DRB3 exon 1, the translation product of which constitutes a part of the ARS in MHCII heterocomplexes. High heterozygosity values support discrimination between one or two allele chimeras and three or four allele chimeras.

Conclusions

Our results suggest that germ cell chimerism is at most inconspicious. The possibility of germ cell chimerism cannot be ruled out, although it has been proven that it is not a prominent phenomenon. The fair possibility that DRB3ms is not polymorphic enough to reveal the chimerism remains open.

Specific markers for bovine PGCs are still lacking and the intravascular cells morphologically and histochemically resemble PGCs seen in sections of bovine embryos (Jost and Prépin 1966, Wartenberg 1983, Ohno 1965, Wrobel 1998). However, they cannot be interpreted as such. Thus there is a possibility that the simple retrospective STR-PCR technique set up here will shed light on germ cell biology in the bovine species.

STR-PCR appeared as a comparatively potent method for extracting indications of chimerism. This far we have been using only a single marker; DRB3ms. Although this marker is an informative one based on the low level of homozygosity detected, some improvement of the resolution is still needed. Other polymorphic mss will be used. The first choice, DRBP1, could enable accurate quantification of any chimerism to be found using linked SNPs in SNP-PCR. STR analysis of the offspring of possibly germ cell chimeric bulls should show paternal inheritance of more than two alleles. In the case of more informative X-chromosomal markers two allele analysis could suffice for the determination of chimerism. Deducing the origin of the alleles from the sire could be done if the marker is polymorphic enough for differentiating the parental genotypes and if the dams are also analysed. Typing the progeny would also exclude the somatic cells in semen, although somatic cells were very rare in our microscopy. Isolation of the sperm cells from the rare somatic cells of semen would require a sensitive method, such as flow cytometry.

The chimerism of only three of the 12 freemartins and their twins analysed would have been recognised by STR-PCR because of unexpected sharing of alleles between the twins or homozygosity in this small pool. These 12 cases showed substantial levels of chimerism in *in situ* experiments. Analysing additional markers will hopefully improve the sensitivity and reliability of the method.

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